

DATA EVALUATION RECORD

GLYPHOSATE

Study Type: OCSPP 890.1550, Steroidogenesis Assay

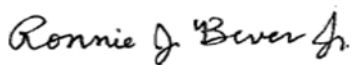
EPA Contract No. EP10H001452

Task Assignment No. 2-57-2012 (MRID 48617005)


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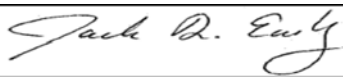
Primary Reviewer:
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Date: 06/12/2012

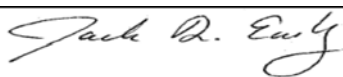
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Date: 06/26/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel.

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: Anwar Y. Dunbar, Ph.D.
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Secondary Reviewer: John Liccione, Ph.D.
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Date: _____

Template version 08/2011

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| DATA EVALUATION RECORD |
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STUDY TYPE: Steroidogenesis Assay (H295R Cells); OCSPP 890.1550

PC CODE: 417300

DP BARCODE: D398693

TXR#: 0053233

CAS No.: 1071-83-6

TEST MATERIAL (PURITY): Glyphosate (not reported)

SYNONYMS: N-(phosphonomethyl)glycine

CITATION: Hecker, M., Hollert H., Cooper, R., *et al.* (2011) The OECD validation program of the H295R steroidogenesis assay: phase 3. Final inter-laboratory validation study. MRID 48617005. Published: Environ. Sci. Pollut. Res. (2011) 18:503-515.

SPONSOR: Not applicable

TEST ORDER #: CON-417300-23

EXECUTIVE SUMMARY: The purpose of this study was to validate the use of a standardized steroidogenesis assay as detailed in *OECD Guideline for the Testing of Chemicals: Draft Proposal for a New Guideline 4XX – The H295R Steroidogenesis Assay* (available on-line at <http://www.oecd.org/dataoecd/56/11/44285292.pdf>). In this validation study, 28 chemicals were selected as a screen for potential effects of endocrine-disrupting chemicals on the production of testosterone (T) and 17 β -estradiol (E2). These chemicals were selected based on their known or suspected endocrine activity, or lack thereof, and included inhibitors and inducers of different potencies as well as positive and negative controls. These chemicals were selected and approved by the OECD Validation and Management Group for Non-Animal Testing (VMG NA). Glyphosate was one of the chemicals evaluated. A total of seven laboratories from the USA, Denmark, Germany, Japan, Hong Kong, and Canada, each with different levels of experience in conducting the H295R steroidogenesis assay, were invited to participate in this validation study. Inclusion of laboratories with different levels of proficiency in conducting the assay was essential to evaluate the completeness of the test protocols and their transferability. Each laboratory was assigned a random code number (1–7) as part of the study. However, part way through the study, two of the seven laboratories decided to cease their participation in the validation studies. Thus, with the exception of the QC exposure data, only the data for the remaining five laboratories that completed the validation studies are presented (Labs 1, 2, 3, 4, and 6). One laboratory evaluated all 28 chemicals, and one other laboratory (#4) also evaluated glyphosate. The laboratories were not identified.

In this steroidogenesis assay (MRID 48617005), H295R cells cultured *in vitro* in 24-well plates were incubated with glyphosate (purity and lot # not provided) at seven concentrations between 0.0001 and 100 μ M (specific concentrations not reported) for 48 hours in triplicate for three independent experiments. The test chemical's vehicle was not identified. The presence or absence of precipitation and/or cytotoxicity was not reported. A Quality Control (QC) plate was run concurrently with each independent run of a test chemical plate to demonstrate that the assay responded properly to positive control agents at two concentrations; positive controls included the known inhibitor (prochloraz) and inducer (forskolin) of estradiol and testosterone production. T and E2 levels were measured using radioimmunoassays or ELISA; responses of the QC plates measured by these assays were confirmed by LC-MS (at Lab 1).

The report stated that with a few exceptions, all of the laboratories met the key quality performance parameters for conducting the H295R assay protocol. The report stated that two laboratories demonstrated that glyphosate exposure does not affect testosterone or estradiol levels in this assay; however, data were not presented.

The assay **satisfies** the EDSP Tier 1 Test Order requirements for a Steroidogenesis assay (OCSPP 890.1550).

COMPLIANCE: Signed and dated GLP Compliance and Quality Assurance statements were not provided in this published article.

I. MATERIALS AND METHODS

The following information was obtained from the cited article (MRID 48617005). Additionally, the cited article stated that a standardized H295R steroidogenesis assay protocol was developed and presented as a proposed draft guideline (<http://www.oecd.org/dataoecd/56/11/44285292.pdf>). The reviewers assume that this protocol was followed without deviation, as the point of this study was the validation of this protocol. These assumptions apply not only to the methodology, but the recommended criteria and required protocol compliance of the results as well. Consequently, some of the materials and methods from this report come from the OECD protocol referenced by the hyperlink.

- A. **MATERIALS**: A total of 28 chemicals were selected in this study to validate the H295R steroidogenesis assay as a screen for potential effects of endocrine-disrupting chemicals on the production of T and E2. These chemicals were selected based on their known or suspected endocrine activity, or lack thereof, and included inhibitors and inducers of different potencies as well as positive and negative controls. Where possible, the test set of chemicals was harmonized with those used in other steroidogenesis assays currently under development or in validation [e.g., the Registration, Evaluation, Authorization and Restriction of Chemical substances (REACH) program]. These chemicals were selected and approved by the OECD VMG NA. Glyphosate was one these chemicals.

1. **Test Facility:**

Location:

Not reported

USA, Denmark, Germany, Japan, Hong Kong, and Canada (laboratories from seven countries were initially involved, but two decided to cease participation; the seven laboratories were not identified)

Study Director:

Not reported

Other Personnel:

Not reported

Study Period:

Not reported

2. **Test Substance:**

Description (molecular weight):

Glyphosate
White crystalline powder (169.07)

Batch # (expiration date):

Not reported

Purity:

Not reported

Solubility:

Not reported; water soluble (1.01 g/100 mL at 20°C)

Vapor pressure:

Not reported; $<1 \times 10^{-5}$ Pa at 25°C

Stability:

Not reported

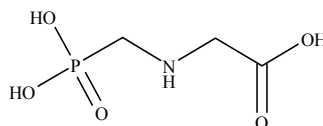
Storage conditions:

Not reported

CAS #:

1071-83-6

Structure:



3. **Positive Control:**

Description (molecular weight):

Forskolin

White powder (410.50)

Source:

Not reported

Lot # (expiration date):

Not reported

Purity:

Not reported

Solubility (in solvent):

Soluble at tested concentrations in DMSO

Storage conditions:

Room temperature

CAS #:

66575-29-9

4. **Negative Control:** Prochloraz
 Description (molecular weight): White powder (376.67)
 Source: Not reported
 Lot # (expiration date): Not reported
 Purity: Not reported
 Solubility (in solvent): Soluble at tested concentrations in DMSO
 Storage conditions: Room temperature
 CAS #: 67747-09-5
5. **Solvent/Vehicle Control:** Not reported (DMSO listed in the proposed draft guideline)
6. **Stock Medium:** Dulbecco's modified Eagle's medium/nutrient mixture F12
 Ham with 15 mM HEPES, sodium bicarbonate,
 ITS+Premix, and 2.5% Nu-Serum (assumed by reviewer)
 Source: Not reported
 Lot # (expiration date): Not reported

7. **Test Cells:** H295R human adrenocortical carcinoma cells (ATCC CLR-2128) were cultured for a minimum of four to five passages to ensure sufficient basal E2 production (cell age was not to exceed ten passages). Cells were incubated in the stock medium at 5% CO₂ and 37°C for approximately 24 hours prior to exposure.

The following performance criteria were met (indicated by an "x"):

| | |
|---|---|
| x | Cell passage identifier. Cell Passage #: Not reported |
| x | Cells frozen down at passage 5 |
| x | Frozen cells cultured for 4 additional passages |
| x | Total number of passages does not exceed 10 |

- B. **METHODS:** A total of seven laboratories from the USA, Denmark, Germany, Japan, Hong Kong, and Canada, each with different levels of experience in conducting the H295R steroidogenesis assay, were invited to participate in this validation study. Inclusion of laboratories with different levels of proficiency in conducting the assay was essential to evaluate the completeness of the test protocols and their transferability. Each laboratory was assigned a random code number (1–7) as part of the study. However, part way through the study, two of the seven laboratories decided to cease their participation in the validation studies. Thus, with the exception of the QC exposure data, only the data for the remaining five laboratories that completed the validation studies are presented (Labs 1, 2, 3, 4, and 6). One laboratory evaluated all 28 chemicals, and one other laboratory (#4) also evaluated glyphosate. The laboratories were not identified.

1. **Pre-Test Information:** The report stated that laboratories were required to demonstrate competence in performing all of the procedures that are part of the H295R steroidogenesis assay prior to testing chemicals. The QC that was part of the actual conduct of the assay to allow for the evaluation of the assay performance during each experiment also served as a benchmark for determining laboratory competence prior to the initiation of chemical testing. Prior to initiation of the actual exposure experiments, each chemical was tested for potential interference with the hormone detection system used. This was of particular relevance for antibody-based assays such as enzyme-linked immunoassays (ELISAs) and radio immunoassays (RIAs), because it has been previously shown that some chemicals can interfere with these tests. The following performance criteria were to be met, and the report

stated that all laboratories met the key quality performance parameters with few exceptions (details not reported):

| System | Parameter | Comparison to/between | T | E2 |
|--------------------------|--|--|-----------|-----------|
| Hormone detection system | Sensitivity | Detectable fold decrease relative to SC | ≥2-fold | ≥2-fold |
| | Precision | CV among replicate measures (absolute concentrations) of the same well for SCs | ≤25% | ≤25% |
| Cell assay | Basal hormone production in SCs | Fold greater than LOQ of hormone detection system | ≥5-fold | ≥2.5-fold |
| | Precision (SCs) | CV among absolute concentrations of replicate wells | ≤30% | ≤30% |
| | Sensitivity (induction @ 10 µM forskolin) | Fold greater than SC | ≥2-fold | ≥7.5-fold |
| | Sensitivity (inhibition @ 3 µM prochloraz) | Fold less than SC | ≥0.5-fold | ≥0.5-fold |

Induction and inhibition refer to the relative change in hormone production after exposure to 10 µM forskolin or 3 µM prochloraz, respectively, in the QC plates

CV Coefficient of variation (%), LOQ limit of quantification, SC solvent control

Resulting data from the pre-test assays were not presented to allow for independent verification.

- a. **Hormone Assay Interference Test:** No data from the hormone assay interference test were provided.
 - b. **Hormone Extraction:** No data from pre-test hormone extraction were provided.
 - c. **Laboratory Proficiency Test:** No laboratory proficiency test data were provided.
2. **Test Solutions:** Details on the preparation of the glyphosate test solution (including the solvent used) were not provided. The presence or absence of precipitation was not reported.
 3. **Cell Plating and Preincubation:** Cells were maintained in the Stock Medium described above. H295R cells were grown for five passages, frozen in liquid nitrogen, then thawed and cultured for at least four or five additional passages prior to use in the assay. The cells were plated into wells of a 24-well cell culture plate at a density of approximately 200,000 to 300,000 cells/mL. The cells were then placed into a 5% CO₂ incubator at 37°C for approximately 24 hours prior to chemical exposure. Prior to dosing, the cells were checked microscopically for attachment and proper morphology. Each experiment was repeated three times with exception of Labs 1 and 3, where one and two replicate experiments were conducted per chemical, respectively.
 4. **Exposure:** Cells were exposed for 48 h to seven concentrations between 0.0001 and 100 µM of the test chemical in triplicate. Although these concentrations were not presented in the study report, the concentrations are typically as shown in Table 1.

| TABLE 1. Dosing Schematic for the Exposure of H295R Cells to Glyphosate (Final Concentrations in μM). ^a | | | | | | |
|---|----------|----------|----------|----------|----------|----------|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| A | DMSO | DMSO | DMSO | 0.1 | 0.1 | 0.1 |
| B | 100 | 100 | 100 | 0.01 | 0.01 | 0.01 |
| C | 10 | 10 | 10 | 0.001 | 0.001 | 0.001 |
| D | 1 | 1 | 1 | 0.0001 | 0.0001 | 0.0001 |

a Not included in the study report.

A concurrent QC plate was included with each of the independent runs of the test chemical plates to demonstrate the assay's response to forskolin (an inducer of testosterone and estradiol production) and prochloraz (an inhibitor of testosterone and estradiol production). The QC plate was prepared and dosed in the same manner as the test plate with either forskolin or prochloraz, according to the schematic presented in Table 2.

| TABLE 2. Dosing Schematic for the QC Plate for Positive Controls (Final Concentrations in μM). ^a | | | | | | |
|--|-------------------------------|-------------------------------|-------------------------------|---------------------------------|---------------------------------|---------------------------------|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| A | Blank | Blank | Blank | Blank + MeOH ^b | Blank + MeOH ^b | Blank + MeOH ^b |
| B | DMSO | DMSO | DMSO | DMSO + MeOH ^b | DMSO + MeOH ^b | DMSO + MeOH ^b |
| C | Forskolin 1 μM | Forskolin 1 μM | Forskolin 1 μM | Prochloraz 0.1 μM | Prochloraz 0.1 μM | Prochloraz 0.1 μM |
| D | Forskolin 10 μM | Forskolin 10 μM | Forskolin 10 μM | Prochloraz 1 μM | Prochloraz 1 μM | Prochloraz 1 μM |

a Data were not included in the study report, but were reported in the OECD protocol on page 11.

b MeOH = methanol was added to these wells for 30 minutes at room temperature following medium removal (end of exposure).

Following dosing, the plates were incubated for 48 hours under the conditions previously described. After the 48 hour exposure, each well was examined under the microscope for cell condition (attachment, morphology, degree of confluence) and signs of cytotoxicity. The media was collected from all wells in two equal portions and stored at -80°C until analyzed.

- 5. Cell Viability/Cytotoxicity Assay:** After media removal, cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosman, 1983) or the Live/Dead® variability assay (Invitrogen, Carlsbad, CA). All concentrations, where cell viability was less than or equal to 80%, were excluded from the data analysis.
- 6. Hormone Measurement System:** At the end of the exposure period, the medium was removed from each well, and hormones were generally extracted using ethyl ether; however, the medium was used without extraction in the RIA assay. The other laboratories used the ELISA detection system, with one laboratory confirming the QC plate hormone results using LC-MS analysis. The ELISA and RIA detection systems used commercially available hormone detection kits. The lower limit of quantification (LLQ) was ≤ 100 pg/mL for testosterone and ≤ 10 pg/mL for estradiol.

The following performance criteria were met (indicated by an "x"):

| | |
|---|--|
| x | Method detection limit (100 pg/mL testosterone; 10 pg/mL estradiol) |
| x | Spiked sample recovery acceptable for two concentrations of testosterone and estradiol (mean measured amount from triplicate samples $\leq 30\%$ of nominal concentration) |

| | |
|---|--|
| x | Hormone cross-reactivity (antibody-based assays only; $\leq 30\%$ of basal production of the respective hormone) |
| x | Solvent control within 75% range below maximum response on standard curve |
| x | Test compound tested for interference with measurement system |

- C. **DATA ANALYSIS:** All data were expressed as mean \pm standard error of the mean (SEM). To examine the relative changes in hormone production, results were normalized to the mean solvent control (SC) value for each assay (i.e., each 24-well plate of cells used to test a given chemical), and results were expressed as percent change relative to the SC. Prior to conducting statistical analyses, the assumptions of data normality and variance of homogeneity were evaluated. Normality was evaluated using standard probability plots or the Shapiro–Wilk’s test. If the data were normally distributed or approximated a normal distribution, differences between chemical treatments and SCs were analyzed using one-way analysis of variance (ANOVA) followed by a two-sided Dunnett’s test. If data were not normally distributed, the Kruskal–Wallis test followed by the Mann–Whitney U test were used. Data analysis was conducted using pooled replicate experiments. All statistical analyses were conducted using SYSTAT 11 (SYSTAT Software, Point Richmond, CA). Differences were considered significant at $p < 0.05$.

II. RESULTS

- A. **TEST COMPOUND:** Data were not presented. Two labs demonstrated that the testosterone and estradiol levels in glyphosate-treated cells were similar to SC. These results were in stated to be in agreement with *in vivo* (fish) data¹. No additional information was provided.
- B. **CYTOTOXICITY:** The presence or absence of cytotoxicity in the glyphosate-treated cells was not reported.
- C. **QC PLATE:** The report stated that with a few exceptions, all of the laboratories met the key quality performance parameters for conducting the H295R assay protocol. It is not clear if this statement was referring to the concurrent QC plates, the Pre-Test, or both. However, the results provided were shown in Figure 1 of the study report on page 507 (copied below).

¹ Soso, AB, Barcellos, LJG, Ranzani-Paiva, MJ, *et al.* (2007) Chronic exposure to sub-lethal concentration of a glyphosate-based herbicide alters hormone profiles and affects reproduction of female Jundi’a (*Rhamdia quelen*). Environ. Toxicol. Pharm. **23**:308–313.

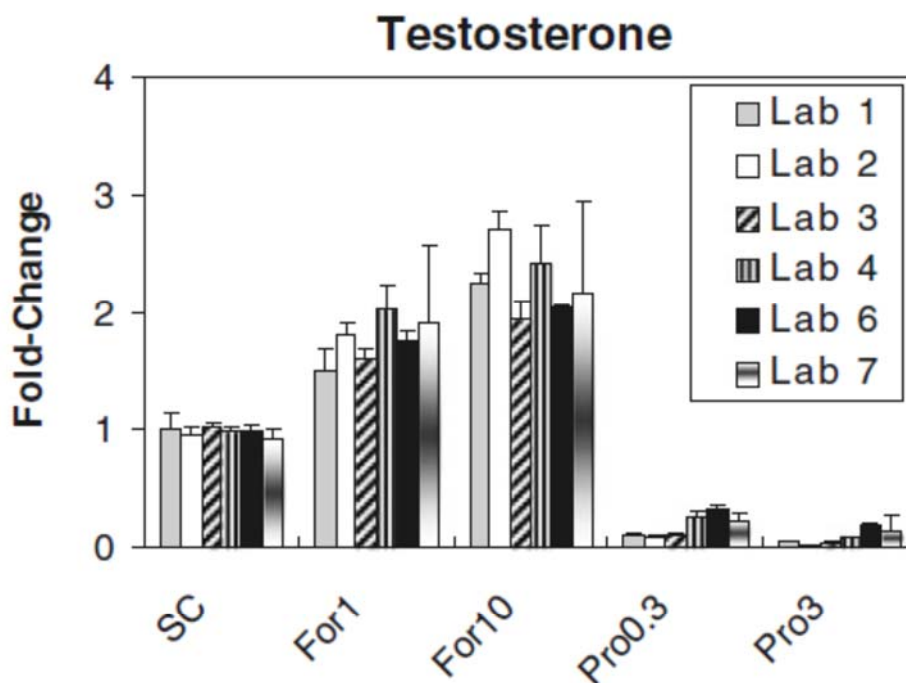
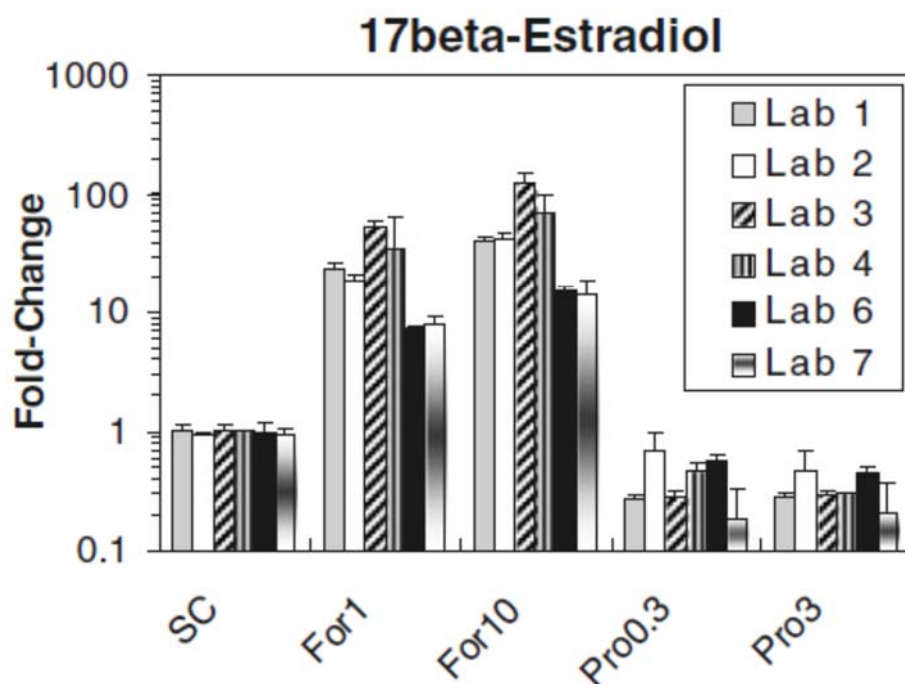


Fig. 1 Comparison of changes in the concentrations of testosterone (T) and estradiol (E2) relative to the solvent controls (SC=1) in the QC plates among laboratories (Lab). For1=1 μ M Forskolin; For10=10 μ M Forskolin; Pro0.3=0.3 μ M Prochloraz; Pro3=3 μ M



Prochloraz. Error bars = $1 \times$ standard deviation. *Bars* represent means of four independent experiments. (Lab 5: only T data from two experiments.)

III. DISCUSSION AND CONCLUSIONS

- A. **INVESTIGATOR'S CONCLUSIONS:** Glyphosate exposure did not affect testosterone or estradiol levels in this assay.
- B. **AGENCY COMMENTS:** Glyphosate was evaluated by two laboratories as part of the OECD validation program of the H295R steroidogenesis assay. Both laboratories reported that glyphosate exposure does not affect testosterone or estradiol levels in this assay.
- C. **STUDY DEFICIENCIES:** In lieu of detailed reporting of the methodology, many assumptions were made regarding the conduct of the assay according to the OECD guideline.